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Multi-Laboratory Evaluation of a Novel Lateral Flow Immunochromatographic Assay for Confirming Isolation of *Mycobacterium bovis* from Veterinary Diagnostic Specimens

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Running Head: Novel *M. bovis*-specific lateral flow device

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(Abstract)

A novel lateral flow immunochromatographic device (LFD) was evaluated in several veterinary diagnostic laboratories. It was confirmed to be specific for *Mycobacterium bovis* and *M. caprae* cells. The performance of the novel LFD was assessed relative to the confirmatory tests routinely applied after culture (spoligotyping or qPCR) in each laboratory; liquid (MGIT or BacT/Alert) and/or solid (Stonebrink, Coletsos or Lowenstein-Jensen) cultures were tested. In comparison to spoligotyping of acid-fast positive MGIT cultures, percentage agreement between positive LFD and spoligotyping results was excellent in two UK laboratories (97.7-100%), but lower in the Spanish context (76%) where spoligotyping was applied to MGIT cultures previously confirmed to be positive for *M. tuberculosis* complex (MTBC) by qPCR. Certain spoligotypes of *M. bovis* and *M. caprae* were not detected by the LFD in Spanish MGIT cultures. Compared to qPCR confirmation, the percentage agreement between positive LFD and qPCR results was 42.3% and 50% for BacT/Alert and MGIT liquid cultures, respectively, and for solid cultures ranged from 11.1-89.2%, depending on solid medium employed (Coletsos 11.1%, Lowenstein-Jensen 55.6%, Stonebrinks 89.2%). Correlation between the novel LFD and BD MGIT TBc ID test results was excellent when 190 MGIT cultures were tested (r = 0.9791; $P<0.0001$), with the added benefit that *M. bovis* was differentiated from another MTBC species in one MGIT culture by the novel LFD. This multi-laboratory evaluation has demonstrated the novel LFD's potential utility as a rapid test to confirm isolation of *M. bovis* and *M. caprae* from veterinary specimens following culture.

Keywords: *Mycobacterium bovis*, *Mycobacterium caprae*, lateral flow immunochromatographic assay, detection specificity, detection sensitivity, veterinary diagnostics

Bovine tuberculosis (bTB), caused primarily by *Mycobacterium bovis*, is endemic in many countries and constitutes a significant economic burden to the agricultural industries (1,2,3). Eradication of bTB is currently one of the biggest challenges facing the cattle industry worldwide and despite intensive eradication efforts over decades, bTB continues to be a problem with global perspective (4,5). Amongst the plethora of factors identified as constraints to eradication are the limitations of existing diagnostic tests (6). Diagnosis of bTB is time consuming, and is compounded in some cases by the presence of non-tuberculosis mycobacteria (NTM) which represent a large and diverse population of mycobacteria which may interfere with diagnosis (7). In addition, although *M. bovis* is the main aetiological agent that causes tuberculosis in domesticated cattle and other wildlife and domesticated species, a very closely related species, *Mycobacterium caprae*, also causes a significant proportion of bTB cases in some European countries (8). Differentiation of NTM from organisms that cause bTB is currently only possible by nucleic acid amplification methods, such as PCR and spoligotyping, which are specific but are technically challenging and require sophisticated instrumentation making them expensive. More rapid, specific and sensitive detection and/or confirmatory methods for *M. bovis* that could potentially replace the currently used non-specific ZN stain and the expensive molecular based techniques are urgently required to expedite accurate diagnosis and reduce cost.

Mycobacterial culture is still regarded as the 'gold standard' technique for diagnosis of bTB (9), despite the fact that it is slow and cultures are sometimes subject to overgrowth by contaminants. Culture and identification techniques for *M. bovis* and *M. caprae* from veterinary specimens are not globally standardised, so consequently a range of both liquid and solid culture media are employed in veterinary diagnostic laboratories worldwide. The time taken to isolate these species by culture can be up to 12-14 weeks, and subsequent tests needed to confirm and speciate an isolate (Ziehl-Neelsen (ZN) staining and microscopy, PCR or qPCR, GenoType MTBC test, or spoligotyping) require additional time, cost and staff training and effort. A rapid test to confirm isolation of *M. bovis*, rather than other members of the *M. tuberculosis* complex (MTBC) or an NTM in suspect positive liquid

or solid cultures is currently lacking. A novel rapid lateral flow, immunochromatographic (LFD) test to detect *M. bovis*, recently developed at Queen's University Belfast (10), may represent such a confirmatory test.

Lateral flow, immunochromatographic tests are an inexpensive, quick and simple-to-use format to visually detect a target of choice (11). Several such tests for detection of *M. bovis,* or MTBC species more generally, are available commercially. These detect either serum antibodies to *M. bovis* (BrockTB Stat-Pak® assay or DPP® CervidTB assay, both Chembio Diagnostic Systems, Inc., Medford, NY), or the MPT 64 antigen secreted by members of the MTBC, including *M. bovis*, in liquid culture (BD MGIT TBc Identification Test, Becton, Dickinson and Company, NJ; SD Bioline TB Ag MPT 64, Standard Diagnostics, Inc., Gyeonggi-do, Republic of Korea; Capilia TB-Neo kit, TAUNS Laboratories, Inc., Shizuoka, Japan). The commercially available MPT 64-based antigen detecting immunochromatographic tests have been shown to be highly reliable for rapid identification of MTBC organisms and as such are considered good alternatives to biochemical and molecular assays (12). However, none of these tests is able to distinguish *M. bovis* from other MTBC species, so the recently developed novel antibody-based LFD test is unique in this respect. In the human clinical TB laboratory setting, the commercially available LFD tests (named above) are being used to differentiate MTBC species from NTM, in order to confirm isolation of MTBC from sputum cultures. According to the MGIT™ Procedure Manual (13), the number of *M. tuberculosis* cells present in a MGIT™ culture whenever it signals positive on the MGIT™ 960 instrument is 10^5-10^6 CFU/ml, which is higher than the limit of detection of the novel LFD (10). Therefore, in the veterinary TB laboratory setting, where the MGIT™ liquid culture system is also used, the novel LFD could potentially be used to quickly confirm isolation of *M. bovis* in liquid cultures of bovine lymph nodes (or other animal specimens) without the need for acid-fast staining and molecular techniques such as spoligotyping or real-time PCR.

The overall aim of this study was to determine if the novel LFD would be applicable in the veterinary laboratory setting to confirm isolation of *M. bovis* from diagnostic samples.

The objectives of the study were to: (i) evaluate the specificity of the novel LFD for *M. bovis*; (ii) assess the performance of the novel LFD relative to current culture confirmation approaches used in veterinary TB laboratories; (iii) evaluate the compatibility of the LFD with three different liquid mycobacterial culture media (BD MGIT, BioMerieux BacT/ALERT, and Trek Diagnostics VersaTREK Myco); and (iv) evaluate the performance of the novel LFD relative to the commercially available BD MGIT™ TBc ID test for confirming presence of *M. bovis* in MGIT cultures in the veterinary diagnostic laboratory setting. Following initial evaluation of the LFD at Queen's University Belfast (QUB) and in the statutory TB culture laboratory at Veterinary Sciences Division, Agri-Food and Biosciences Institute for Northern Ireland (AFBI), evaluation of the novel LFD was extended to four other veterinary TB laboratories.

MATERIALS AND METHODS

Participating laboratories. The following laboratories were involved in the study: TB Immunology Laboratory, Veterinary Sciences Division, Agri-Food and Biosciences Institute, Stormont, Belfast, Northern Ireland (AFBI); TB Laboratory, Animal and Plant Health Agency Starcross, Exeter, England (APHA); Laboratoire Départemental d'Analyse & de Recherche, Service Analyses Agriculture et Vétérinaire, Dordogne, France (DORDOGNE); Servicio de Micobacterias, Centro de Vigilancia Sanitaria Veterinaria, Madrid, Spain (VISAVET); Laboratorio Regional de Sanidad Animal, León, Spain (LRSA); and Laboratory of Immunology, Embrapa Gado de Corte, Campo Grande, MS, Brazil (EMBRAPA); The Animal TB Research Group, Stellenbosch University, South Africa (SUN).

Description of novel lateral flow device (LFD). A prototype LFD was developed by researchers at QUB in collaboration with Forsite Diagnostics Limited (now trading as Abingdon Health), York, England, as part of a United Kingdom Department of Environment, Food and Rural Affairs project (Defra SE3271). It is an antibody-based antigen detection test, as defined by Office International des Epizooties (OIE) (14). The device comprises of a nitrocellulose membrane strip with a Test line (T) of an *M. bovis*-specific polyclonal IgG

antibody produced by QUB personnel and a Control line (C) of a commercially available anti-mouse IgG antibody, and employs gold nanoparticles coated with an *M. bovis*-specific monoclonal IgG antibody, originally produced by AFBI personnel, as the labelled detector reagent. For Intellectual Property (IP) reasons no further details about the antibodies involved can be provided. Additional detail on the development and optimisation of the *M. bovis*-specific LFD is available elsewhere (15). The prototype LFDs used in this study (approx. 1300 tests) were produced by Forsite Diagnostics Limited, and then distributed by QUB to participating laboratories by courier service, along with the required running buffer, blocking reagent and instructions for use.

LFD specificity checks. Participating laboratories were requested to select cultures for LFD specificity testing, to reflect as broad a range of MTBC and NTM species as were available to them, and as representative a collection of strains for each species as possible. Prior specificity checks on the novel LFD had determined that it did not cross react with a range of Gram positive and Gram negative bacteria that may occur in cattle lymph nodes and human sputum (unpublished data). The majority of strains tested were field isolates whose identification had been confirmed by a molecular method (spoligotyping, RD4/RD9 analysis and/or 16S rRNA gene sequencing). The cultures tested had generally been freshly sub-cultured in/on various culture media (dictated by usual laboratory practice), including three different liquid media (MGIT from Becton Dickinson, BacT/ALERT from BioMerieux, versaTREK Myco from Thermofisher) and three different solid agar media (Coletsos, Lowenstein-Jensen and Stonebrink), before testing on the LFD. However, older MGIT and solid mycobacterial cultures were tested in some of the laboratories. When the LFD was used to test liquid cultures, 1 ml of culture was centrifuged at 14,000 rpm for 15 min and the pellet resuspended in 100 µl freshly prepared KPL Detector™ Block (KPL, Inc., Gaithersburg, MA, USA), before 80 µl was transferred to the sample well of the LFD. When used to test solid cultures, a single colony was thoroughly emulsified in 100 µl KPL blocking solution and then 80 µl was transferred to the sample well of the LFD. In both instances, the LFD result was recorded after 15 min at room temperature, interpreted as follows: *M. bovis*

positive if two lines were visible; *M. bovis* negative if only a C line was visible; and 'Invalid test' result if only a T line was present, or neither T nor C lines were present. In the latter case, if additional LFDs were available, the LFD test was repeated with a 10-fold dilution of the resuspended pellet of that particular culture to determine if that yielded a valid result.

Assessment of the performance of the novel LFD applied to liquid and solid cultures of veterinary specimens in comparison to current confirmation approaches. In order to assess the ability of the novel LFD to confirm isolation of *M. bovis***,** each laboratory tested selected liquid and/or solid cultures, whichever were available, and provided LFD results along with results obtained using their currently applied confirmatory approach (ZN staining and spoligotyping, or qPCR) to QUB. Variable numbers of cultures of tissues from different animals (cattle, badgers, wild boar, deer, goats) were tested in each laboratory.

At the statutory veterinary TB laboratory in Northern Ireland (AFBI) tissue specimens from skin test reactor cattle, or bovine lymph nodes detected at routine slaughter, are chemically decontaminated and cultured in MGIT liquid culture medium and on Lowenstein-Jensen (LJ), Middlebrook 7H11 and/or Stonebrink slopes. Confirmation of isolation of *M. bovis* is carried out by spoligotyping of DNA from acid-fast positive MGIT cultures or from suspect colonies on solid media. In an initial assessment, 240 MGIT cultures were selected to be tested, comprising of 40 each of six different categories of MGIT culture and ZN outcome commonly encountered in this laboratory: (1) MGIT positive, ZN 3+; (2) MGIT positive, ZN 2+; (3) MGIT positive, ZN 1+; (4) MGIT positive, ZN 'atypical'; (5) MGIT positive, ZN negative; (6) MGIT negative, ZN not done. LFD testing did not commence until all samples for all categories became available, so these MGIT cultures were not tested in 'real-time', i.e. as they indicated growth positive on the MGIT 960 machine or completed the 56 day incubation period. Rather, they were removed from the MGIT 960 instrument, ZN stained to permit culture categorisation, and then kept in an incubator until all required cultures became available. The 240 MGIT cultures were blind coded before the QUB post-doc tested each culture on the LFD. Subsequently, an additional 105 MGIT cultures were

196 tested in ‘real-time’ by AFBI personnel as soon as possible after they indicated positive on
197 the MGIT 960 instrument, or as they finished the 56 d incubation on the MGIT system.
198 The statutory veterinary TB laboratory in England (APHA Starcross) uses a similar
199 confirmatory approach to AFBI; spoligotyping is used to confirm isolation of *M. bovis* in liquid
200 cultures, but isolates on solid agar are reported on the basis of colony morphology with
201 confirmation by spoligotyping at the herd breakdown level only. At APHA, 190 MGIT cultures
202 were selected for LFD testing, categorised on the basis of solid and liquid culture outcomes
203 and ZN result as follows: (A) solid and MGIT positive, ZN positive; (B1) solid negative, MGIT
204 positive, ZN positive; (B2) solid negative, MGIT positive, ZN negative; and (C) solid and
205 MGIT negative, ZN not done.
206 In contrast to AFBI and APHA TB test procedures, at the laboratories in Spain
207 (VISAVET and LRSA), France (DORDOGNE) and Brazil (EMBRAPA), MGIT liquid culture,
208 or BacT/ALERT and versaTREK Myco liquid culture, and solid culture on Coletsos, LJ (with
209 pyruvate) or Stonebrink media are variously employed after decontamination of veterinary
210 specimens, and real-time qPCR methods, which vary by country, are routinely used to
211 confirm the isolation of MTBC in liquid culture; spoligotyping would only be carried out on
212 some cultures. In the Brazilian laboratory, two qPCR methods targeting TbD1 (16) and
213 Rv2807 (17) are employed. In the French laboratory, a qPCR targeting IS6110 is employed
214 for diagnosis currently (18). The Spanish laboratories use an unpublished qPCR method
215 targeting the region between Rv0953c-Rv0954 for MGIT liquid cultures (Elena Alonso,
216 LRSA, personal communication) and spoligotyping for isolates on solid LJ medium.
217 **Comparison of the performance of novel LFD and commercially available BD**
218 **MGIT™ TBc ID test applied to MGIT™ cultures**. Personnel at the APHA laboratory tested
219 the 190 MGIT cultures of veterinary specimens mentioned above by the BD MGIT™ TBc ID
220 test (Becton Dickinson, Sparks, Maryland, USA) in parallel with the novel LFD test. This
221 permitted direct comparison of the performance of the two LFDs.
222 **Statistical analysis of results.** For each laboratory the percentage of spoligotyping- or
223 qPCR- confirmed cultures that tested positive by the LFD was calculated for liquid or solid

cultures, as appropriate. Correlation between numbers of samples positive by LFD and by qPCR at DORDOGNE, VISAVET/LRSA and EMBRAPA was assessed by Spearman's rank correlation coefficient. Cross-tabulation of the novel LFD and BD TBc ID test results for the 190 MGIT cultures tested at APHA permitted determination of a Kappa statistic, as a measure of the agreement between the two tests, which was interpreted according to Landis and Koch (22). Fisher's Exact Test was also performed on the results for each LFD and confirmed MGIT culture results (i.e. after spoligotyping of acid-fast positive liquid cultures) to permit estimation of detection sensitivity and specificity of each LFD applied to MGIT cultures of veterinary specimens. Statistical tests were performed using GraphPad Instat® 3.10 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Specificity of the novel LFD. Pure cultures of MTBC species (all except *M. canetti*) plus a broad range of NTM were tested on the novel LFD. Some species were available as liquid cultures and some as solid cultures within the various participating laboratories. In the EMBRAPA laboratory only solid cultures were available, some of which were freshly prepared and others were described as 'old and difficult to emulsify'. LFD test results for all pure liquid and solid cultures tested are summarised in Table 1 and Table 2, respectively. In total 85 different strains of *M. bovis*, 41 of *M. tuberculosis* (all of human origin) and 1-4 isolates of 29 different NTM (including both type and field strains) were tested across all of the laboratories. The *M. bovis* isolates tested as part of the specificity evaluation were predominantly from cattle, but also included isolates from goats and wild boar (VISAVET and LRSA), lions, mongooses, baboons, civet, hyena and buffalo (SUN), and badgers (DORDOGNE). The *M. bovis* strains tested represent a broad range of different spoligotypes (some information is provided in footnotes of Tables 1 and 2).

Results in Tables 1 and 2 indicate that the novel LFD gave a positive result with *M. bovis, M. caprae* and *M. bovis* BCG, and, also with the two strains of *M. pinnipedii* tested. Overall, 41 (95.3%) of the 43 *M. bovis* strains tested as liquid cultures yielded a positive LFD

result. In contrast, when solid cultures were tested only 60 (75%) of the 80 *M. bovis* strains tested yielded a positive result (Table 2). Some false negative or 'invalid' LFD results for confirmed *M. bovis* strains (including *M. bovis* BCG) and *M. caprae* strains were encountered, particularly with colonies from Coletsos (9/14 strains in DORDOGNE tested LFD negative), LJ (4/9 and 5/10 strains in DORDOGNE and VISAVET, respectively, tested LFD negative) and Stonebrinks slopes (8/60 strains at EMBRAPA tested LFD negative) (Table 2).

Performance of novel LFD applied to liquid and solid cultures compared to confirmation by spoligotyping. At AFBI, MGIT cultures exhibiting growth are ZN stained and only spoligotyped if acid-fast cells are observed to be present. When 160 MGIT cultures categorised on the basis of growth and ZN result were tested at AFBI, the LFD indicated the presence of *M. bovis* in 118 (98.3 %) of 120 ZN positive MGIT cultures (scored 1+, 2+ or 3+; categories 1-3), in 20 (50 %) of 40 MGIT cultures recorded as having an 'atypical' ZN result (category 4), and in 4 (10%) of 40 ZN negative MGIT cultures (category 5). ZN negative MGIT positive cultures are not routinely spoligotyped at AFBI, so the potential presence of an *M. bovis* spoligotype in the ZN negative MGIT cultures that tested LFD positive cannot be excluded. All 40 growth negative MGIT cultures (category 6) tested negative by the LFD. When positive LFD results were compared with the spoligotyping outcome for the MGIT cultures, there was 100% agreement between an LFD positive culture and the presence of an *M. bovis* spoligotype (Table 3). When AFBI personnel subsequently tested 105 MGIT cultures in 'real-time', i.e. as soon as possible after they had flagged positive on the MGIT 960 instrument, there was still 100% agreement between an LFD positive culture and the presence of an *M. bovis* spoligotype, but, as was the case for the categorised MGIT cultures (detailed above), an additional two ZN negative cultures tested LFD positive.

At APHA, when 190 routine MGIT cultures of bovine lymph tissue samples categorised on the basis of growth on the MGIT system and ZN result were tested, 90 of the 103 MGIT cultures in categories A and B1 were confirmed to contain MTBC by spoligotyping, and 89 contained *M. bovis*. The novel LFD indicated the presence of *M. bovis*

in 88 (98.9%) of these 89 *M. bovis* positive MGIT cultures (Table 3). The other two LFD negative but MTBC positive MGIT cultures did contain *M. bovis* in one case (so a false negative LFD result) but another MTBC species in the other case (so a true negative LFD result). None of 87 ZN negative MGIT cultures (categories B2 and C) tested LFD positive.

The Spanish laboratories (VISAVET and LRSA) tested MGIT cultures of tissues from four different animal species (cattle, goats, deer and wild boar), in contrast to most other participating laboratories, where cattle tissues were principally cultured. For the purposes of this study, spoligotyping was carried out on the qPCR positive MGIT cultures to confirm if *M. bovis* or *M. caprae* were present. Overall, of the 50 VISAVET/LRSA MGIT cultures where either *M. bovis* or *M. caprae* were identified to be present by spoligotyping, 39 MGIT cultures tested LFD positive (76% agreement, Table 3). When the *M. bovis* and *M. caprae* spoligotypes present in the LFD negative MGIT cultures were considered, it became apparent that MGIT cultures containing certain spoligotypes of *M. bovis* (SB0121, SB0134, SB0152, SB0295 and SB0339) and *M. caprae* (SB0157, SB0415 and SB0416), isolated from cattle and goat specimens, had not been detected by the novel LFD (Table 4).

Performance of novel LFD applied to liquid and solid cultures compared to qPCR confirmation of MTBC isolation. Real-time qPCR, rather than ZN staining and spoligotyping of acid-fast cultures, was routinely being used in the non-UK laboratories to confirm the isolation of MTBC from veterinary specimens after liquid and/or solid culture, although different qPCR methods were being used in the three laboratories. Results of liquid culture testing at VISAVET/LRSA and DORDOGNE are shown in Figure 1, and for solid culture testing at EMBRAPA in Figure 2. In these figures results are presented for LFD and qPCR as the number of cultures LFD positive when the C_T value of the MGIT culture, or the emulsified suspect colony, was 'x', and no. of cultures yielding a C_T value of 'x' by qPCR, respectively. Correlation between numbers of cultures testing positive by the two tests was assessed using Spearman's rank correlation coefficient (GraphPad Instat® 3.10). For VISAVET/LRSA results (Figure 1A) there was found to be significant correlation between numbers of cultures testing LFD and qPCR positive for MGIT™ cultures yielding C_T values

from 17-26 (Spearman's r = 0.9271; *P*=0.0003), however for cultures yielding C_T values >26 there was no significant correlation (Spearman's r = 0.1164; *P*=0.7185) between the two tests. Similarly, for the DORDOGNE results (Figure 1B) there was found to be significant correlation between numbers testing LFD and IS6110 qPCR positive for BacT/ALERT cultures yielding C_T values from 14-27 (Spearman's r = 0.830; *P*=0.0003), however for cultures giving C_T values >27 there was no significant correlation (Spearman's r = 0.1164; *P*=0.7185) between the two tests. These results indicate that the limit of detection of the qPCR methods is lower than that of the LFD, meaning that qPCR applied to liquid cultures will detect higher numbers of MTBC positive cultures than testing by the LFD, however, as the qPCR methods employed are neither *M. bovis* nor *M. caprae* specific the presence of other MTBC species in some of these samples cannot be ruled out.

VISAVET/LRSA and DORDOGNE liquid culture test results were also analysed in terms of percentage agreement between positive LFD and qPCR results (Table 3). The percentage agreement between positive LFD and qPCR results at VISAVET/LRSA was 50 % (39 of 78 cultures); which was lower than agreement between spoligotyping and LFD results (76 %, 38 of 50 confirmed *M. bovis* or *M. caprae* positive cultures). When C_T values of the 50 cultures confirmed by spoligotyping were considered, all except three had C_T values $\leq$ 30, which is consistent with the trend illustrated in Figure 1A. DORDOGNE was the only participating laboratory using the BacT/Alert liquid culture system rather than the MGIT culture system, and it was the only laboratory to report 'invalid' LFD results (i.e. no C line in the presence or absence of a T line) when testing liquid cultures. As a consequence of this, percentage agreement between LFD and qPCR positive results for liquid cultures of veterinary specimens tested at DORDOGNE was lowest at 42.3% (Table 3).

EMBRAPA results exclusively represented testing of emulsified colonies from solid cultures of bovine specimens by two different confirmatory qPCRs and LFD (Figure 2). There was significant correlation between numbers of cultures testing LFD positive and TbD1 qPCR positive (Spearman's r = 0.973, *P*=0.0001). For qPCR targeting TbD1, C_T values of emulsified colonies from 60 agar slants ranged from 12.5–25.0. Of these, 52

(91.2%) of 57 emulsified colonies with C_T values ranging from 12.5–22.5 tested LFD positive, and three with C_T values >23 tested LFD negative (Figure 2A). Similar results were observed with the second qPCR targeting Rv2807 applied to emulsified colonies. There was significant correlation between numbers of cultures testing LFD positive and Rv2807 qPCR positive (Spearman's r = 0.967, *P*=0.0001). For Rv2807 qPCR, C_T values of emulsified colonies from 60 agar slants ranged from 12.64–25.94. Of these, 52 (88.1%) of 59 emulsified colonies with C_T values ranging from 12.64–23.0 tested LFD positive, and one with C_T value of 25.94 tested LFD negative (Figure 2B). Both fresh and old confirmed *M. bovis* cultures on Stonebrink slopes were tested by EMBRAPA; 89.2 and 82.6% of fresh and old solid cultures, respectively, were confirmed to be *M. bovis* by the LFD (Table 5). No 'invalid' LFD results were reported by EMBRAPA (just some difficulties in emulsifying colonies in KPL buffer on occasion); in contrast to the DORDOGNE laboratory where 8 of 9 Coletsos cultures and 4 of 9 LJ cultures yielded an 'invalid' result (Table 5). The latter resulted in only 11.1 and 55.6% of solid cultures being confirmed as *M. bovis* by the LFD in the French context (Table 5).

Correlation between the results of the novel LFD and the commercially available BD MGIT™ TBc ID test. A total of 190 MGIT™ cultures of bovine specimens were tested in parallel by the novel LFD and BD MGIT™ TBc ID tests at APHA. Correlation between results was assessed using Spearman's rank correlation coefficient, which indicated significant correlation between results obtained with the two LFDs (r = 0.9791; *P*<0.0001). LFD results are presented as separate 2x2 contingency tables relative to confirmed culture result (on the basis of spoligotyping) for the two LFDs in Table 6. There were two MGIT cultures with discordant results with the two LFD tests – one culture was MTBC positive but confirmed *M. bovis* negative by spoligotyping, so correctly tested negative by the novel LFD but positive by the BD MGIT™ TBc ID test, and the other culture was confirmed *M. bovis* positive by spoligotyping and tested falsely negative by the novel LFD but correctly positive by the BD MGIT™ TBc ID test (Table 6). There were an additional two MGIT™ cultures that yielded negative results by both LFD tests when different results were expected; in one case both

tests should have yielded positive results because *M. bovis* was confirmed to be present by spoligotyping, and in the other case the BD MGIT™ TBc ID test should have yielded a positive result because an MTBC species other than *M. bovis* was indicated by spoligotyping. When the commercial and novel lateral flow tests were used to confirm the presence of *M. bovis* in MGIT cultures of veterinary specimens, the detection specificity and sensitivity of both LFD tests were comparable - 1.000 and 0.978, respectively, for the novel LFD, and 0.990 and 0.989, respectively, for the BD MGIT TBc ID test.

DISCUSSION

Evaluation of the novel LFD test was carried out in multiple veterinary diagnostic laboratories located in different geographic regions, processing specimens from a range of animal species, and using differing bTB diagnostic algorithms,in the hope that the results of the study would provide a wide-ranging assessment of its potential utility as a quick and easy end point test to confirm isolation of *M. bovis* from animal specimens in the veterinary diagnostic context. Veterinary diagnostic laboratories in different countries adopt differing approaches for confirming isolation of MTBC after culture of animal specimens. The bTB diagnostic algorithm adopted is dependent mostly on the current prevalence of tuberculosis in the country, which dictates whether testing is being carried out for disease surveillance, control or eradication purposes. For example, bTB is endemic in Northern Ireland (herd prevalence 7.15% and animal incidence 0.66%, (20)) and England and Wales (herd prevalence ~7.8%, (21,22)), so in the UK context confirmation of isolation of *M. bovis* from bovine specimens is achieved by colony morphology on solid agar or ZN staining and spoligotyping of DNA extracted from acid-fast positive liquid cultures. Spoligotyping facilitates epidemiological studies and provides transmission data required for control and eradication in the UK context. In contrast, herd prevalences of bTB in cattle in France (~0.05%, Jean-Louis Moyen, DORDOGNE, personal communication) and Spain (2.81%, (23)) are lower, but there is a recognised threat of reintroduction of the disease due to wildlife ‘spill-back’ (24,25). In these contexts a more sensitive qPCR approach to confirming

392 the isolation of *M. bovis* (or *M. caprae* since this species is also encountered) in liquid and
393 solid cultures has been adopted; ZN staining is not routinely employed and all isolates would
394 not necessarily be spoligotyped. Similarly, in Brazil, a recent epidemiological survey
395 indicated the prevalence of TB in infected cattle ranged from 0.035 to 1.3% in the 13 States
396 surveyed (26), so qPCR confirmation methods are adopted.

397 A wide range of NTM and MTBC strains from both solid and liquid cultures was
398 tested to evaluate the specificity of the novel LFD. The results (Tables 1 and 2) confirmed
399 that the LFD was specific for *M. bovis* in the broadest sense, since *M. bovis*, *M. caprae* and
400 *M. bovis* BCG all gave rise to both positive T and C lines on the device. Detection of *M.*
401 *caprae* by the novel LFD would be viewed as beneficial, since this MTBC species is the main
402 aetiological agent of tuberculosis in goats, but also in cattle in certain countries. For
403 example, in Spain around 7% of bTB cases in cattle are due to *M. caprae* (8), and in some
404 central/eastern European countries TB infection in cattle is only due to *M. caprae* (27).
405 However, *M. caprae* strains with different RD4 deletions have been isolated in parts of
406 Europe (ML Boschiroli, personal communication; 28); which may explain some of the
407 negative LFD results obtained for colonies of 5 of 10 *M. caprae* strains from solid
408 LJ/pyruvate medium and 5 of 8 confirmed *M. caprae* positive MGIT liquid cultures tested by
409 VISAVET/LRSA (Table 2). Alternatively, certain spoligotypes may not be detectable by the
410 LFD. In phylogenetic terms, amongst the MTBC species, *M. pinnipedii* and *M. microti* are
411 closest to *M. bovis*, *M. caprae* and *M. bovis* BCG (27). A positive result on the novel LFD
412 was obtained with the single liquid and single solid cultures of *M. pinnipedii* tested, however,
413 this *Mycobacterium* sp. is primarily isolated from seals and is rarely encountered in cattle or
414 food animals (29). *M. microti* can sometimes be encountered in cats, badgers or wild boars
415 in the UK and French contexts, but rarely in food animals (30) so it is advantageous that the
416 two liquid cultures and one solid culture of *M. microti* tested negative with the novel LFD.

417 Once the detection specificity of the LFD had been demonstrated, the diagnostic
418 performance of the LFD was assessed by comparing LFD results with those of current
419 nucleic acid-based confirmatory tests (spoligotyping and qPCR) employed in the various

laboratories. Few issues were encountered by personnel using the LFD for the first time, or in interpretation of LFD results. Some false negative, or invalid (no C-line), LFD results occurred, mainly when testing BacT/ALERT liquid cultures or emulsified confirmed *M. bovis* colonies from Coletsos and LJ solid media in the DORDOGNE laboratory (Tables 3 and 5). When testing colonies it may be that cells were not adequately disaggregated and so clumped cells were too big to pass along the LFD to reach the T and C lines to generate a positive result. An alternative explanation could be that too many cells passing along the LFD may have quenched or prevented binding of cell/particle complexes at the T and/or C lines leading to false negative or invalid test outcomes (Dene Baldwin, Abingdon Health, personal communication). In the DORDOGNE laboratory some BacT/ALERT liquid cultures that initially gave a negative or invalid LFD result when retested after 10-fold dilution yielded a valid result (i.e. presence of a C-line) or a clear positive result; which appeared to confirm two things - that too much biomass was the cause of false negative results, and that higher numbers of *M. bovis* were present in positive BacT/ALERT cultures than in positive MGIT cultures. EMBRAPA personnel mentioned difficulties in emulsifying some of the older *M. bovis* colonies, however, they did not report any 'invalid' LFD results. It is clear that some more optimisation of the LFD test procedure in relation to its application to confirm the identity of suspect colonies from solid culture media and in liquid culture systems other than BD MGIT 960 system would be needed. This would include optimising the density of a colony suspension relative to the universally used McFarland scale before application to the LFD.

The results of this study clearly show that the novel LFD performed better relative to culture confirmation by spoligotyping than culture confirmation by qPCR. In the UK laboratories (AFBI and APHA) percentage agreement between spoligotyping and LFD results for liquid MGIT cultures was 97.7-100% (Table 3). Therefore, the LFD could potentially be used instead of ZN and spoligotyping for routine and rapid confirmation of *M. bovis* isolation in cultures of veterinary specimens once growth is indicated by the MGIT 960 culture system in the UK context. However, since spoligotyping is also carried out to provide

448 valuable epidemiological information (31-35), it is probably unlikely that the LFD test would
449 replace spoligotyping completely in these laboratories. Some extra *M. bovis* positive MGIT
450 cultures were indicated by LFD testing compared to ZN staining, so if the LFD were adopted
451 in the UK context this could potentially more efficiently direct spoligotyping efforts than ZN
452 staining of MGIT cultures currently does. In the Spanish context percentage agreement
453 between the spoligotyping and LFD results was lower at 76%. Results suggest that isolates
454 of certain spoligotypes of both *M. bovis* (SB0121, SB0134, SB0152, SB0295 and SB0339)
455 and *M. caprae* (SB0157, SB0415 and SB0416) were not being detected by the LFD (Table
456 4). However, it should be noted that VISAVET/LRSA did not apply ZN staining prior to
457 spoligotyping of qPCR positive MGIT cultures; which may also have contributed to the lower
458 percentage agreement figure.

459 Various qPCR methods are routinely employed to confirm the isolation of MTBC
460 species in liquid or solid cultures from veterinary diagnostic specimens in the non-UK
461 laboratories (DORDOGNE, VISAVET/LRSA and EMBRAPA). It is evident from the results of
462 this study that the novel LFD is less sensitive than qPCR. This is not a surprising finding
463 given that the limit of detection of the LFD is 10^4-10^5 *M. bovis* cells/ml of sample (10) and the
464 limit of detection of qPCR methods for MTBC is generally much lower than this (18); for
465 example, the limit of detection of the French IS6110 qPCR is 3 genomic units (Jean-Louis
466 Moyen, DORDOGNE, personal communication). Results presented in Figures 1 and 2
467 suggest that the limit of detection of the novel LFD is whatever number of *M. bovis* cells a C_T
468 value in the mid to high 20s corresponds to. This number may differ depending on the qPCR
469 method employed, but is likely to be a reasonably high number of *M. bovis*, which would
470 concur with the limit of detection of the LFD previously determined using spiked faeces and
471 dilutions of *M. bovis* cultures at QUB (10). Unfortunately, information on what number of *M.*
472 *bovis* cells equates to a C_T value in the mid-20s was not obtainable from any of the three
473 laboratories concerned; in these laboratories the qPCR methods were being used
474 qualitatively and not for quantification.

During this study, liquid and/or solid cultures of veterinary specimens from a variety of animal species were tested in the participating laboratories. In the AFBI, APHA and DORDOGNE laboratories, liquid cultures of principally cattle and some badger specimens were tested by the novel LFD. In the EMBRAPA laboratory exclusively cattle cultures isolated on solid media were tested. However, in the VISAVET and LRSA laboratories MGIT liquid cultures tested were of cattle, goats, deer and wild boar specimens. The LFD seemed to perform less well on cattle and goat liquid cultures in the Spanish context; several *M. bovis* and *M. caprae* spoligotypes were seemingly not detectable by the LFD (Table 4). In Spain around 7% of bTB cases in cattle are due to *M. caprae* (8), and TB cases in goats in Spain can be caused by either *M. bovis* or *M. caprae* (36). It has previously been reported that 62 different spoligotypes were identified amongst MTBC isolates collected from wild ungulates and livestock in Spain (37). Given the diversity of MTBC spoligotypes that are encountered in the Spanish veterinary diagnostic context, perhaps it is the case that certain spoligotypes of *M. bovis* and *M. caprae* occurring in Spanish animals are not detectable by the combination of antibodies (originally generated using *M. bovis* AF2122/97, a UK cattle strain with spoligotype SB0140) employed on the LFD. This may also explain the negative LFD results obtained for 5 (50%) of 10 pure cultures of *M. caprae* tested from solid medium (Table 2) and 12 (24%) of 50 MGIT cultures of Spanish veterinary specimens with low qPCR C_T values that were confirmed to contain either *M. bovis* or *M. caprae* by spoligotyping (Table 4).

Commercially available antibody-based LFD tests, which target MPT64 or MPT70 secreted antigens, not whole cells, and detect MTBC species more generally have been . The BD MGIT TBc ID immunochromatographic assay has been comprehensively evaluated in human clinical TB laboratories to speciate mycobacterial isolates to the level of MTBC or NTM (38-42). The BD LFD and other similar commercially available MPT64-based LFD tests perform very well in the clinical TB context and have been found to be good alternatives to biochemical and molecular assays for identification to the level of MTBC species in cultures of respiratory specimens (12). To our knowledge, there have been no

previous reports of the BD MGIT TBc ID test being evaluated for use in the veterinary TB diagnostic setting. In this latter context, *M. bovis* and *M. caprae* (in certain geographic regions), rather than *M. tuberculosis,* are the MTBC species most commonly isolated, and confirmation of isolation of *M. bovis* or *M. caprae* is achieved by spoligotyping, or of MTBC isolation more broadly by a specific qPCR. A quicker and cheaper confirmatory test, that is able to confirm isolation of *M. bovis* or *M. caprae* but doesn't require expensive equipment, or a lot of staff training or time, should be an attractive proposition for a veterinary TB laboratory. During this study the performance of the novel LFD was found to be comparable to that of the BD MGIT TBc ID test for confirming isolation of *M. bovis* in MGIT liquid cultures of veterinary specimens at APHA in terms of detection sensitivity and specificity. However, the extra differentiation between *M. bovis* and other species of the MTBC possible using the novel LFD would potentially be a more attractive proposition for the APHA laboratory than simply confirming isolation of MTBC.

In conclusion, evaluation of the novel LFD in multiple veterinary TB laboratories in various parts of the world has demonstrated that the novel LFD could find application in the veterinary diagnostic setting to confirm isolation of *M. bovis* or *M. caprae* (depending on geographical context) in liquid cultures (assuming sufficient cell numbers are present), and also to test suspect colonies from solid culture media. The LFD was shown to have excellent specificity for this purpose, and its unique ability to differentiate *M. bovis* and *M. caprae* from other MTBC and NTM was clearly demonstrated; in contrast to other commercially available antibody-based LFD tests. The novel LFD possessed sufficient sensitivity to confirm the isolation of *M. bovis* or *M. caprae* in liquid cultures once they indicated positive on the MGIT 960 or BACT/Alert culture systems, with a few exceptions.

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682 **Table 1.** Multi-laboratory assessment of the specificity of the novel LFD by testing pure cultures of *M. tuberculosis* complex and non-
683 tuberculous *Mycobacterium* spp. grown in liquid culture media. All liquid cultures tested signalled positive on the respective culture systems
684 and, therefore, contained high numbers of mycobacteria (>10^6 CFU/ml) at time of testing.

Test *Mycobacterium* sp.	QUB 7H9/OADC	DORDOGNE		VISAVET/LRSA MGIT™	SUN MGIT™	No. (%) LFD positive/ total no. cultures tested
		BacT/ALERT®	versaTREK® Myco			
	No. LFD positive / No. tested					
***M. tuberculosis* complex:**						
M. bovis	6/6*	4/4	8/8	-	23/25	41/43 (95.3)
M. caprae	-	1/1	1/1	-	-	2/2 (100)
M. bovis BCG	1/1	-	-	-	-	1/1 (100)
M. pinnipedii	-	-	-	1/1	-	1/1 (100)
M. africanum	-	0/1	0/1	-	-	0/2 (0)
M. microti	-	0/1	0/1	-	-	0/2 (0)
M. tuberculosis	0/1	0/1	0/1	-	0/41	0/44 (0)
Non-tuberculous mycobacteria:						
M. abscessus	-	-	-	-	0/1	0/1 (0)
M. asiaticum	-	-	-	-	0/1	0/1 (0)
M. avium subsp. avium	0/1	0/1	0/1	0/1	-	0/4 (0)
M. avium subsp. paratuberculosis	0/10	0/2	0/2	-	-	0/14 (0)
M. chelonae	-	-	-	-	0/1	0/1 (0)
M. columbiense	-	-	-	0/1	-	0/1 (0)
M. diernhoferi	-	-	-	-	0/1	0/1 (0)

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M. elephantis	-	-	-	-	0/1	0/1 (0)
M. fortuitum	0/1	-	-	0/1	0/1	0/3 (0)
M. gordonae	0/1	-	-	-	-	0/1 (0)
M. hassiacum	-	-	-	-	0/1	0/1 (0)
M. hiberniae	0/1	-	-	-	-	0/1 (0)
M. interjectum	-	-	-	-	0/1	0/1 (0)
M. intracellulare	0/1	-	-	0/1	-	0/2 (0)
M. kansasii	0/1	1/1 (v. weak)**	0/1	0/1	-	1/4 (25)
M. lentiflavum	-	-	-	-	0/1	0/1 (0)
M. marinum	0/1	-	-	0/1	-	0/2 (0)
M. moriokaense	-	-	-	-	0/1	0/1 (0)
M. nonchromogenicum	-	0/1	0/1		-	0/2 (0)
M. paraffinicum	-	-	-	-	0/1	0/1 (0)
M. parascrofulaceum	-	-	-	-	0/1	0/1 (0)
M. peregrinum	-	-	-	0/1	-	0/1 (0)
M. porcinum	-	-	-	-	0/1	0/1 (0)
M. scrofulaceum	0/1	-	-	-	-	0/1 (0)
M. simiae	-	-	-	-	0/1	0/1 (0)
M. smegmatis	0/1	-	-	0/1	-	0/2 (0)
M. terrae	0/1	-	-	-	-	0/1 (0)
M. vulneris	-	-	-	-	0/1	0/1 (0)
M. xenopi	0/1	-	-	-	-	0/1 (0)

-, not tested

* *M. bovis* spoligotypes tested – SB0140, SB0129, SB0273, SB0142, SB0263 and SB0145, according to Mbovis.org database.

** very weak T-line observed, but slightly increasing blocking buffer concentration prevented this false positive result.

708 **Table 2.** Multi-laboratory assessment of the specificity of the novel LFD involving pure
709 cultures of *Mycobacterium tuberculosis* complex and non-tuberculous *Mycobacterium* spp.
710 grown on different solid culture media (Coletsos, Lowenstein-Jensen (LJ) or Stonebrink).
711 Data represent number of solid cultures LFD positive of the total number of solid cultures
712 tested in each case.

713

Test *Mycobacterium* sp.	DORDOGNE		VISAVET/LRSA	EMBRAPA	No. (%) LFD positive/ total no. cultures tested
	Coletsos medium	Lowenstein-Jensen (LJ) medium	LJ/pyruvate medium	Stonebrink medium	
***M. tuberculosis* complex:**					
M. bovis	3/11*	5/9*	-	52/60	60/80 (75.0)
M. caprae	1/1	-	5/10**	-	6/11 (54.5)
M. bovis BCG	1/2*	-	-	-	1/2 (50)
M. pinnipedii	1/1	-	-	-	1/1 (100)
M. africanum	0/1	-	-	-	0/1 (0)
M. microti	-	-	0/1	-	0/1 (0)
M. tuberculosis	0/1	-	-	-	0/1 (0)
Non-tuberculous mycobacteria:					
M. avium subsp. avium	0/1	-	-	0/1	0/2 (0)
M. chelonae	-	-	-	0/1	0/1 (0)
M. fortuitum	-	-	-	0/1	0/1 (0)
M. kansasii	0/1	-	-	-	0/1 (0)
M. nonchromogenicum	0/1	-	-	-	0/1 (0)

714 -, not tested

715 *Dordogne laboratory reported Control (C) line absent, with and/or without positive Test (T)
716 line, so LFD result was 'Invalid'.

717 ** *M. caprae* spoligotypes tested by VISAVET/LRSA: SB0416, SB0418, SB2205, SB2281,
718 according to Mbovis.org database.

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Table 3. Performance of novel LFD test applied to liquid mycobacterial cultures (BD MGIT or BioMerieux BacT/Alert) of specimens from a range of animal species in comparison with usual confirmatory test(s) applied in multiple veterinary diagnostic laboratories.

Laboratory (Country)	Liquid culture system employed (No. cultures tested)	Animal species represented	Confirmatory test(s) routinely applied to liquid cultures	No. cultures confirmed *M. bovis* or MTBC positive[3]	No. cultures LFD positive	% of confirmed cultures testing LFD positive
AFBI (N. Ireland, UK)	MGIT (240 categorised[1])	Cattle,	Ziehl-Neelsen stain +	132	141	100[4]
	MGIT (105 real-time[2])	badgers	spoligotyping	38	40	100[4]
APHA (UK)	MGIT (190 categorised[1])	Cattle, badgers	Ziehl-Neelsen stain + spoligotyping	89	88	98.9
DORDOGNE (France)	BacT/Alert (52)	Cattle, badgers	qPCR	52	22	42.3
VISAVET/LRSA (Spain)	MGIT (78)	Cattle, goat, wild boar, deer	qPCR	78	39	50.0
			Spoligotyping[5]	50	38	76.0

[1] Cultures were categorised before LFD testing, as described in Materials and Methods section, and were held in an incubator until all cultures in each category became available before LFD testing commenced.

[2] Cultures were tested as soon as possible after they indicated growth positive on the MGIT 960 instrument, with minimal additional incubation time before LFD testing commenced.

[3] Confirmation as *M. bovis* when spoligotyping applied, but otherwise confirmation to MTBC level by qPCR.

[4] The LFD detected 9 and 3 extra *M. bovis* positive MGIT cultures than spoligotyping for the categorised and real-time cultures, respectively.

[5] Spoligotyping identified all the strains as MTBC but only 50 were identified as *M. bovis* or *M. caprae* with the spoligotyping profile. A breakdown of the spoligotyping results by animal species is provided in Table 4.

Table 4. Breakdown of spoligotyping results for 78 qPCR positive MGIT cultures tested at VISAVET/LRSA and for the 39 MGIT cultures that tested LFD positive.

Origin of specimen	*M. bovis* spoligotype present		*M. caprae* spoligotype present		Unable to assign spoligotype[4]	
	No.	No. LFD +	No.	No. LFD +	No.	No. LFD +
Cattle (n=40)	25	17[1]	1	1	14	1
Goats (n=18)	11	10[2]	7	4[3]	0	0
Wild boar (n=17)	4	4	0	0	13	0
Deer (n=3)	2	2	0	0	1	0

[1] LFD did not detect eight confirmed *M. bovis* positive MGIT cultures containing five different spoligotypes (3 x SB0121, 2 x SB0295, SB0134, SB0339 and SB0152).

[2] LFD did not detect one confirmed *M. bovis* positive MGIT culture containing spoligotype SB0121.

[3] LFD did not detect three confirmed *M. caprae* positive MGIT cultures containing three different spoligotypes (SB0415, SB0416 and SB0157).

[4] Cultures tested MTBC positive by qPCR and spoligotyping, but neither *Mycobacterium* species nor a specific spoligotype could be assigned.

Table 5. Performance of novel LFD test applied to colonies from solid cultures of confirmed *M. bovis* cattle isolates in two veterinary diagnostic laboratories.

Laboratory (Country)	Solid culture medium employed (No. of cultures tested)	LFD result[1]			% of cultures confirmed as *M. bovis* by LFD
		Positive	Negative	Invalid	
		No. of cultures			
DORDOGNE (France)	Coletsos (9)	1	0	8	11.1
	Lowenstein-Jensen (9)	5	0	4	55.6
EMBRAPA (Brazil)	Stonebrink:				
	Fresh sub-cultures (37)	33	4	0	89.2
	Old cultures (23)	19	4	0	82.6

[1] LFD result is 'positive' when both C-line and T-line are visible, 'negative' if only C-line is visible and 'Invalid' if no C-line was present along with a positive T-line.

Table 6. Comparison of the ability of the novel LFD and the commercially available BD MGIT™ TBc ID test to confirm the presence of *Mycobacterium bovis* in 190 MGIT cultures of bovine lymph tissue tested by APHA. Data were analysed by Fisher's exact test and Kappa interrater test.

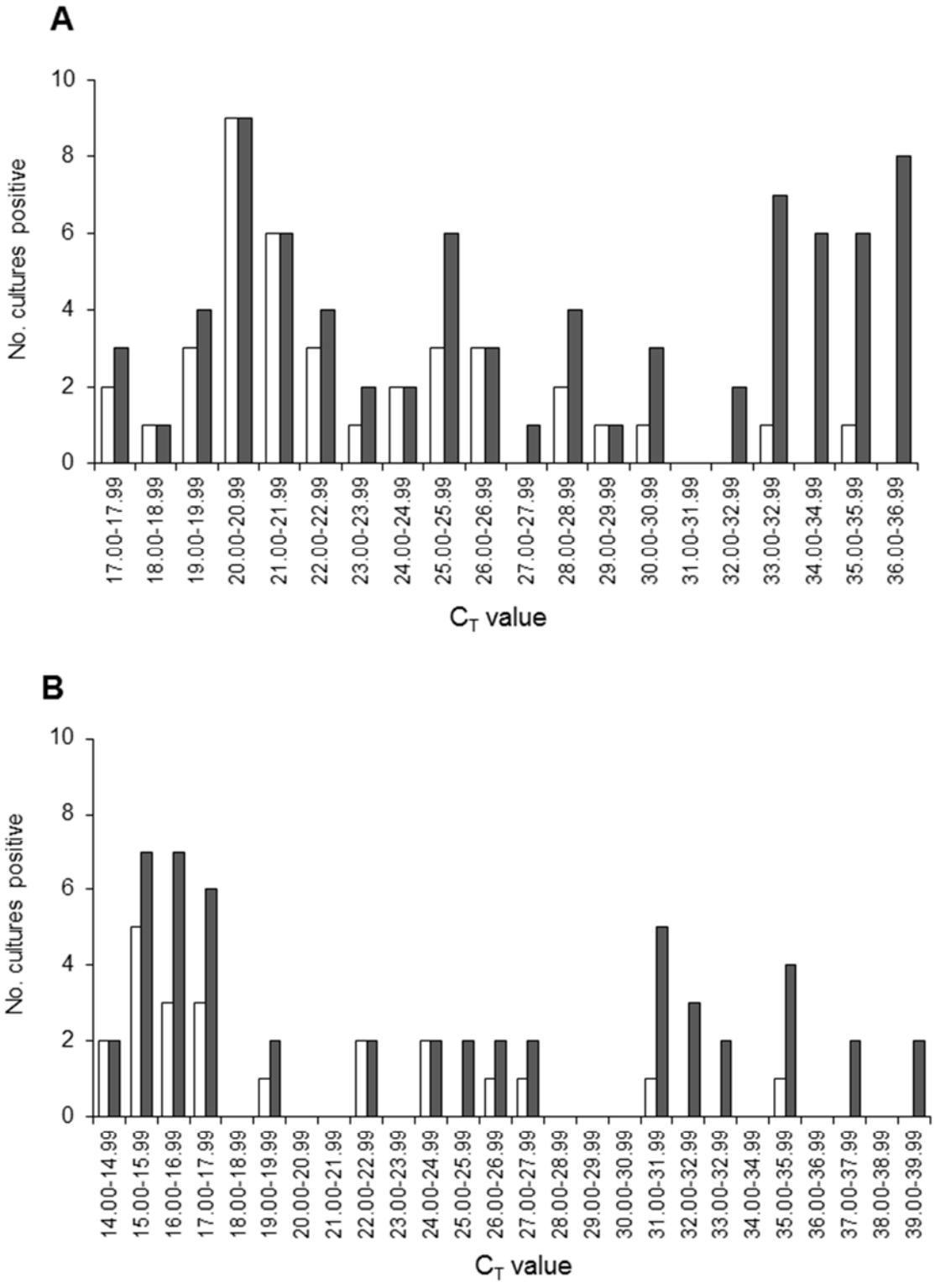
LFD test result	MGIT culture +[1]	MGIT culture -	Kappa statistic (95% CI)	Detection sensitivity (95% CI)	Detection specificity (95% CI)
Novel LFD +	88	0	0.979	0.978	1.000
Novel LFD -	2	100	(0.950-1.000)	(0.922-0.997)	(0.963-1.000)
MGIT TBc ID test +	89	1	0.979	0.989	0.990
MGIT TBc ID test -	1	99	(0.950-1.000)	(0.940-0.999)	(0.946-0.999)

[1] Only recorded as 'Culture +' when presence of *M. bovis* was confirmed by spoligotyping.

Figure legends

Figure 1. Comparison of the numbers of liquid cultures at (A) VISAVET/LRSA (MGIT cultures) and (B) DORDOGNE (BacT/ALERT cultures) testing positive by the novel LFD (light bars) and by MTBC-specific qPCR (and spoligotyping in case of VISAVET/LRSA data) (dark bars).

Figure 2. Comparison of the numbers of confirmed *M. bovis* solid cultures tested at EMBRAPA testing positive by novel LFD (light bars) and two qPCR methods (dark bars): (A) TbD1 qPCR and (B) Rv2807 qPCR.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6
8
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18.00-18.99
19.00-19.99
20.00-20.99
21.00-21.99
22.00-22.99
23.00-23.99
24.00-24.99
25.00-25.99
26.00-26.99
27.00-27.99
28.00-28.99
29.00-29.99
30.00-30.99
31.00-31.99
32.00-32.99
33.00-32.99
34.00-34.99
35.00-35.99
36.00-36.99
C_T value
B
No. cultures positive
0
2
4
6
8
10
14.00-14.99
15.00-15.99
16.00-16.99
17.00-17.99
18.00-18.99
19.00-19.99
20.00-20.99
21.00-21.99
22.00-22.99
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33.00-32.99
34.00-34.99
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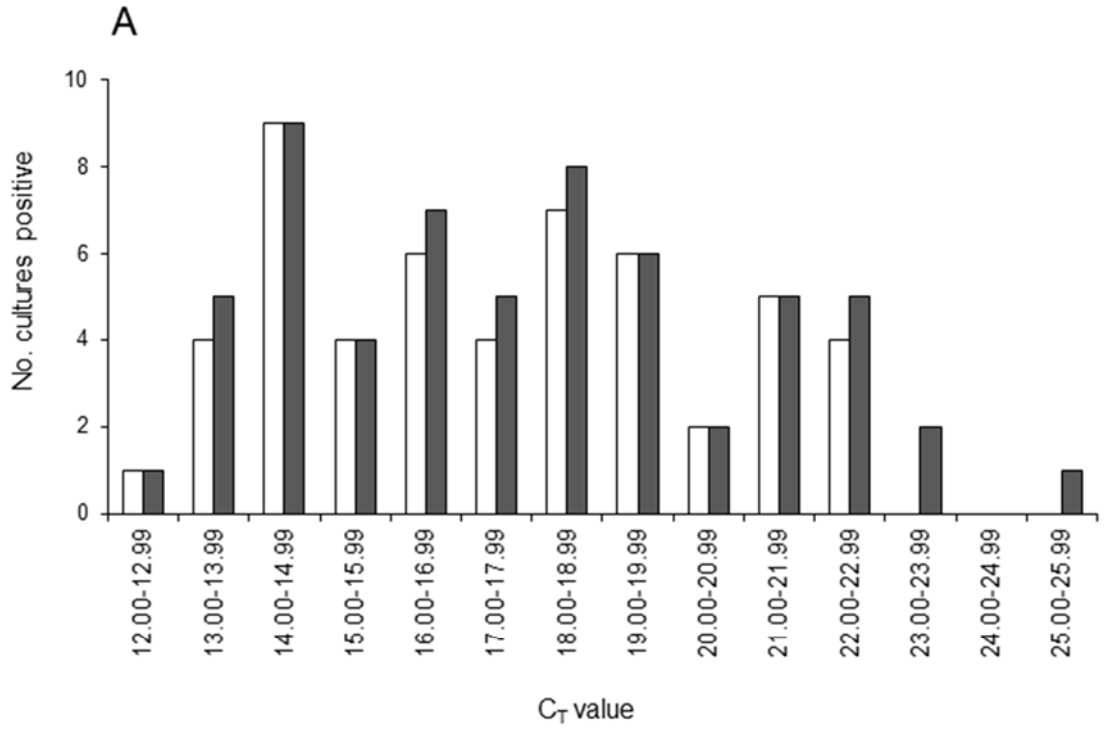
37.00-37.99
38.00-38.99
39.00-39.99
C_T value

25

26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14.00-14.99
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16.00-16.99
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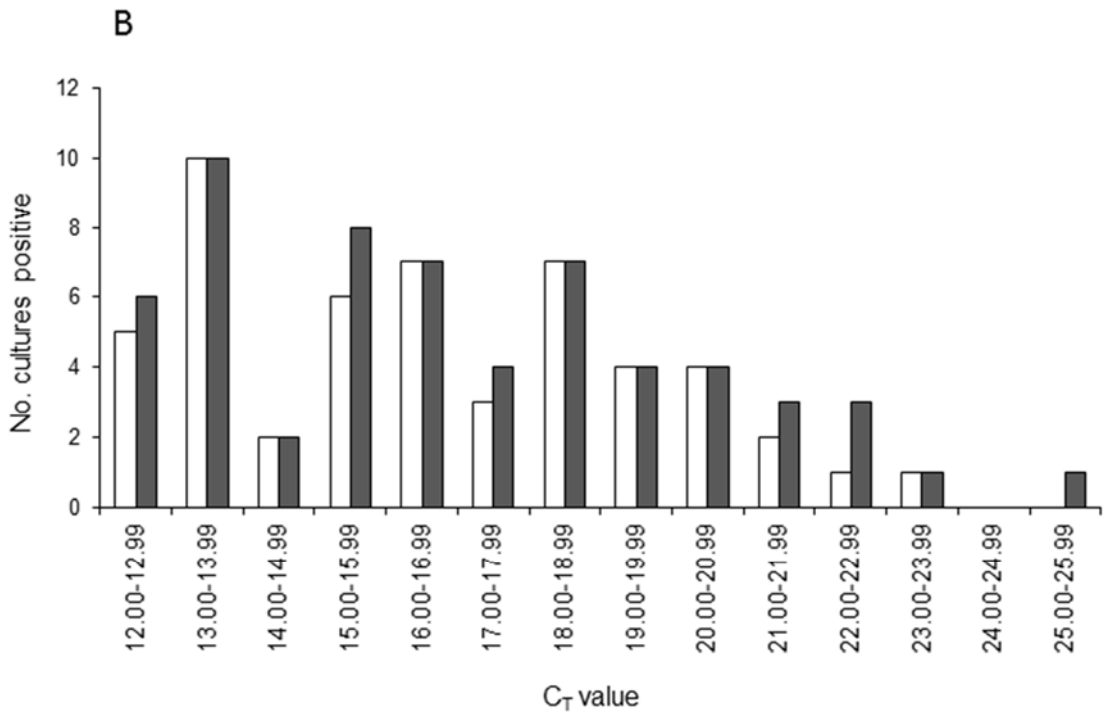
25.00-25.99
C_T value


B
No. cultures positive
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14.00-14.99
15.00-15.99
16.00-16.99
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19.00-19.99
20.00-20.99
21.00-21.99
22.00-22.99
23.00-23.99
24.00-24.99
25.00-25.99
C_T value

27